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Antigen Recognition Is Facilitated by Invadosome-like Protrusions Formed by Memory/Effector T Cells

Peter T. Sage,‡ Laya M. Varghese, † Roberta Martinelli, † Tracey E. Sciuto, ‡ Masataka Kamei, † Ann M. Dvorak, ‡ Timothy A. Springer,§ Arlene H. Sharpe,* and Christopher V. Carman†

Adaptive immunity requires that T cells efficiently scan diverse cell surfaces to identify cognate Ag. However, the basic cellular mechanisms remain unclear. In this study, we investigated this process using vascular endothelial cells, APCs that possess a unique and extremely advantageous, planar morphology. High-resolution imaging revealed that CD4 memory/effect T cells dynamically probe the endothelium by extending submicron-scale, actin-rich “invadosome/podosome-like protrusions” (ILPs). The intimate intercellular contacts enforced by ILPs consistently preceded and supported T cell activation in response to endothelial MHC class II/Ag. The resulting calcium flux stabilized dense arrays of ILPs (each enriched in TCR, protein kinase C-θ, ZAP70, phosphotyrosine, and HS1), forming what we term a podo-synapse. Similar findings were made using CD8 CTLs on endothelium. Furthermore, careful re-examination of both traditional APC models and professional APCs suggests broad relevance for ILPs in facilitating Ag recognition. Together, our results indicate that ILPs function as sensory organelles that serve as actuators of immune surveillance. The Journal of Immunology, 2012, 188: 000–000.

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covery that ECs express MHC class I (MHC-I), MHC class II (MHC-II), and a large number of costimulatory molecules (e.g., CD40, LFA-3, ICOSL, 4-1BB, OX40L, TL1A, PD-L1, but not CD80 and CD86) has led to the controversial hypothesis that endothelium can also function as a type of APC (26–28). Indeed, several studies have demonstrated that endothelium can effectivly restimulate CD4 and CD8 memory/effector, but not naive, T cells (29–33). Critically, when grown in vitro, ECs form virtually planar cell surfaces that are ideal for high spatiotemporal resolution imaging of topological dynamics (34).

This study provides a detailed investigation of the initial events in CD4 and CD8 T cell scanning for Ag. We previously discovered that lymphocytes actively probe the surface of the endothelium by dynamic insertion and retraction of submicron-scale, actin-rich cylindrical protrusions related to invadosomes (35), termed invadosome/podosome-like protrusions (ILPs) (34). These were demonstrated to function in supporting migratory pathfinding (34, 36). In this study, we found that dynamic ILP probing by CD4 and CD8 memory/effector lymphocytes enforces close T cell–EC apposition, which seems to facilitate Ag recognition and TCR signaling. Moreover, T cell activation is sustained through a novel IS architecture dominated by dense arrays of calcium-stabilized ILPs (each enriched in signaling molecules) that we term a podosynapase. Complementary studies with model substrates and professional APCs (B cells and dendritic cells [DCs]) suggest that rather than being a unique feature of T cell–EC ISs, ILPs are achievable acquisition frame rate of this analysis. Data was then sorted into 10-s interval bins.

For selected studies, lymphocytes were preincubated, or lymphocyte and APCs coincubated, with select pharmaceutical agents, including latrunculin A (1 μg/ml; Sigma), BAPTA-AM (40 μM, Sigma), BTP2 (20 μM; Calbiochem), thapsigargin (1 μM Sigma), and calcium-caldulin-dependent kinase II (CAMKII) inhibitor CK59 (50 μM; EMD Biosciences) as indicated. For calcium blockade conditions, T cells were pretreated with BAPTA-AM and BTP2 in imaging buffer containing high (45 mM) potassium. T cells were added to ECs and imaged with additional BTP2 for the duration of imaging.

**Fixed cell imaging**

Fixed cell samples were imaged on an LSM 510 confocal microscope (Zeiss). Samples were fixed and stained for α-Lin (T529/4), ICAM-1 (IC1/11), F-actin (phalloidin), MHC-II (WR18; Abcam), protein kinase C (PKC)-δ, talin (rabbit anti-talin, a gift from Dr. Keith Burridge), CD3 (OKT3; Biotec), CD4 (CD45, or control IgG Ab (10 ng/ml). Fluo-4–labeled lymphocytes were added and imaged by fluorescence and interference reflection microscopy.

**CTL killing assay**

For specific killing assay, murine ECs were labeled with either 0.6 or 0.02 μM CFSE (CFSE− and CFSE+, respectively). The CFSE+ population was pulsed with 1 μg/ml SIINFEKL (OVA257-264) for 20 min. Both CFSE− and CFSE+ populations were plated together in a 1:1 ratio. Activated OTI CD8 T cells were rested for 5 h and added to monolayers in a 3:1 T cell/target cell ratio. Cultures were stained for CD105 to differentiate the ECs from T cells and analyzed by flow cytometry. Specific lysis was calculated by the following equation: 1 − (CFSE−/(CFSE− + CFSE+)) [control] CFSE−/CFSE+ + 100.

**Planar coated-glass APC model**

Coated glass IS models were prepared as described previously (20, 38). In brief, imaging chambers were coated with ICAM-1–Fc (10 ng/ml; R&D Systems) alone or together with anti-CD3, anti-CD43, anti-CD45R, or control IgG Ab (10 μg/ml). Fluor-4–labeled lymphocytes were added and imaged by fluorescence and interference reflection microscopy (IRM).
cell surface biotinylation for 15 min with biotin-NHS-ester (0.5 μg/ml; Thermo Electron). Cells were incubated with SA at 10 μg/ml for 15 min. Cells were then incubated with biotinylated anti-CD3, -CD43, or -CD45 (eBioscience) at 10 μg/ml for 15 min.

**T cell activation by CD3 cross-linking**
Fluo-4–labeled T cells were settled onto uncoated glass chambers, 10 μg/ml anti-CD3 and -CD28 were added to the imaging chambers, and calcium was monitored.

**Transmission electron microscopy**
Transmission electron microscopy of T cell–APC complexes was performed as described previously (34). Fifty randomly selected micrographs were analyzed by enumerating zones of membrane apposition (<20 nm) and correlating them with respect to ILP location.

**Statistical analysis**
Error bars represent SE or SD as indicated. The p values were calculated via unpaired Student t tests in GraphPad Prism. Statistical significance is indicated with p values as follows: ***p < 0.0005, **p < 0.005, *p < 0.05.

**Results**

**Endothelial-presented Ag promotes activation of CD4 memory T cells**
We wanted to elucidate the mechanisms by which memory/effector T cells probe for Ag using ECs as model APCs. Therefore, we isolated natural CD4 memory-like lymphocytes (nTmem) and generated induced/expanded CD4 memory-like lymphocytes (iTmem) from human peripheral blood. The isolated nTmem were CD4+, CD45RO−, and contained both central memory and effector memory T cells (~78 and ~22%, respectively) based on CD62L staining (Supplemental Fig. 1A). The iTmem were slightly more polarized toward the effector memory T cell subtype (~69 versus ~31%; Supplemental Fig. 1B). Monolayers of HLMVECs, HDMVECs, and HUVECs were used as models for the vasculature. In vivo endothelial MHC-II expression is dependent on IFN-γ (26). This was recapitulated in vitro by culturing ECs with exogenous IFN-γ (Supplemental Fig. 1C). Endothelium was additionally activated with TNF-α to promote an inflamed phenotype.
Next, we set up live-cell analysis to concomitantly monitor T cell activation (i.e., calcium flux) and migration on endothelium pulsed with Ag (i.e., bacterial superantigen, a widely used model Ag) (38, 40, 41). In the absence of Ag, nTmem and iTmem fluxed little calcium (Fig. 1A), remained polarized (Fig. 1B), and underwent continuous lateral (Fig. 1C) and transendothelial migration (i.e., “diapedesis”; Fig. 1D). On HLMVECs pulsed with Ag (1 μg/ml SEB and TSST), T cells rapidly fluxed calcium, lost polarity, and arrested migration (Fig. 1A–C). Similar results were found with alternate Ag (e.g., SEE and MAM) presented by HDMVECs or HUVECs (Supplemental Fig. 1D and data not shown). In addition, in the absence of Ag, the majority of iTmem transmigrated within 30 min, whereas in the presence of Ag, diapedesis was delayed by ~30–60 min (Fig. 1D). Thus, the migratory stop signal was transient. iTmem also exhibited Ag-dependent transient migration arrest under conditions of laminar fluid shear flow conditions similar to those found in microvasculature in vivo (2 dyne/cm²; Fig. 1E–G, Supplemental Video 1).

During T cell activation, the transcription factor NFAT translocates from the cytoplasm to the nucleus. Incubation of nTmem on endothelium pulsed with Ag (TSST/SEB) resulted in the formation of stabilized arrays of invadosome-like protrusions (ILPs) (Fig. 2A and B). These arrays were observed on the membrane of T cells in a punctate pattern, forming rings on the endothelium under adherent lymphocytes. The formation of these arrays was dependent on the presence of Ag, as control experiments without Ag did not show similar structures (Fig. 2A and B, control). Similar results were observed with alternate Ag (e.g., SEE and MAM) presented by HDMVECs or HUVECs (Supplemental Fig. 1D and data not shown).

To further analyze the behavior of these ILPs, the distance of individual ILPs from the centroid of the T cell was measured. The data showed that ILPs formed and disappeared in a transient manner, with a mean distance from the cell centroid of 1.9 ± 0.9 μm (Fig. 2E). The change in distance of the ILPs from the cell centroid at the time of formation and disappearance was also plotted, revealing a persistent change in distance during the lifetime of each ILP (Fig. 2F). The observations were supported by statistical analysis, which showed a significant change in distance with p < 0.0005.
Ag-pulsed ECs promoted robust nuclear translocation of NFAT (Fig. 1H, 1I). Significantly, transmigrating nTmem and iTmem retained nuclear NFAT, demonstrating that these cells remained activated (Fig. 1J). NTmem on endothelium that was pulsed with Ag but not pretreated with IFN-γ (and therefore lacked strong MHC-II expression) exhibited little NFAT translocation (Fig. 1I), confirming that the responses to Ag-pulsed endothelium were MHC-II dependent.

To show that T cells achieved complete activation, we conducted studies using a transwell system, in which T cells loaded into an upper chamber migrated across an EC monolayer to reach the lower chamber. Flow cytometry revealed that after migrating across Ag-pulsed ECs, iTmem showed increased surface expression of CD69 and a reduction in CD62L (Supplemental Fig. 1E). In addition, in these nonpolarizing conditions, a subset of the iTmem upregulated IFN-γ expression (Supplemental Fig. 1F, 1G) and increased proliferation (Supplemental Fig. 1H). Collectively, these results demonstrate that endothelium is able to present model Ag in an MHC-II–dependent manner to CD4 memory/effector lymphocytes, which induces a transient delay in diapedesis and T cell activation.

ILP arrays dominate the T cell–EC interface during activation

Next, we investigated the cellular and molecular basis for T cell activation in our endothelial APC model. Previously, we used fluorescent membrane markers (mem-YFP or mem-DsRed) expressed in the ECs to detect topological changes in the plasma membrane (34). In this way, we demonstrated that ~0.5-μm fluorescent rings that formed dynamically on the endothelium under migrating T cells corresponded to cylinder-shaped cell surface invaginations (i.e., “podo-prints”) induced by lymphocyte ILPs. Thus, podo-prints formed on the EC surface served as an indirect but sensitive readout for T cell ILPs (34). In this study, we similarly observed in control settings that podo-prints formed and disappeared

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Ag-stabilized ILPs exhibit a discrete three-dimensional architecture. (A) Ag-stabilized T cell ILPs protrude into the EC surface. Imaging was conducted as in Fig. 2B on HLMVECs coexpressing soluble, cytoplasmic GFP (green) and mem-DsRed (red). (B) Three-dimensional reconstruction from confocal imaging of podo-prints and ILPs. iTmem were incubated for 20 min on activated Ag-pulsed (SEE) HUVECs and then fixed, stained for ICAM-1 (green) and LFA-1 (red), and imaged by confocal microscopy. Sections were digitally reconstructed and projected as three-dimensional renderings. (b) Magnified view of the ILP arrays. (b’) Orthogonal cross section. See also Supplemental Video 5. (C and D) Ag-stabilized ILPs are enriched in actin and talin. Mem-YFP–transfected (C; green) or mem-DsRed–transfected (D; red) HLMVECs were pulsed with Ag (TSST/SEB) and incubated with iTmem, for 5 min and stained for F-actin (C; red) or talin (D; green). (E) Quantitation of ultrastructural depth and width of T cell ILPs. Samples as in (C) were imaged by electron microscopy and ILPs were measured. Data represent mean ± SEM from at least 100 ILPs from at least 20 representative micrographs per condition. (F and G) ILPs enforce close T cell–EC membrane apposition in the absence and presence of Ag. T cell and endothelial nuclei are indicated with red and green overlays, respectively. Arrows and (a) highlight regions of extremely close lymphocyte–EC membrane apposition enforced at the ILP tips. Scale bars, 5 μm (A–D); 500 nm (F, G). *p < 0.05. ns, Not significant.
continuously (with lifetimes of tens of seconds) under the leading edge lamellipodia of migrating iTmem (Fig. 2A, 2C, Supplemental Video 2). Strikingly, when ECs were pulsed with Ag, iTmem rapidly formed dense arrays of podo-prints largely localized at the periphery of the T cell–EC interface under symmetrical lamellipodia (Fig. 2B, example 1, and Supplemental Video 3). A minority (∼20–30%) of the iTmem cells formed rosette-type podo-print arrays that lacked bias for the cell periphery (Fig. 2B, example 2, and Supplemental Video 4). In both cases, podo-prints/ILPs were significantly stabilized (lifetimes of ∼18 min; Fig. 2C) and exhibited limited lateral translocation (0.31 ± 1.5 μm; Fig. 2D–F). After ∼30 min of contact, ILPs began to disappear and T cells initiated transmigration (Supplemental Video 4 and data not shown).

As confirmation that the earlier observations reflected three-dimensional podo-prints in response to T cell ILPs, we cotransfected endothelium with mem-DsRed together with soluble GFP as a marker for cytoplasmic volume (34). This showed individual fluorescent membrane rings of each podo-print, in fact, represented cytosol-displacing invaginations into the EC surface (Fig. 3A). Furthermore, confocal imaging showed that podo-prints were ICAM-1–enriched cylindrical EC invaginations into which T cell ILPs extended (Fig. 3B, Supplemental Video 5). ILPs were enriched in LFA-1 (Fig. 3B), F-actin (Fig. 3C), and talin (Fig. 3D), similarly to invadosomes (35, 36). Analogous structures formed under physiologic shear flow (Supplemental Fig. 2A), with diverse ECs (including HLMVECs [Fig. 3A, 3C, 3D], HUVECs [Fig. 3B], HDMVECs [data not shown]), with iTmem (Supplemental Fig. 2B), and with alternate Ag (Fig. 3B, Supplemental Fig. 2D). Similar structures also formed when previously activated murine OT-II CD4+ T cells were incubated on heart microvascular ECs pulsed with OVA 323–339 peptide Ag (Supplemental Fig. 2E).

To assess the T cell–EC interaction in greater detail, we used transmission electron microscopy. In the absence of Ag, ILPs averaged 430 ± 34 nm in depth and 348 ± 33 nm in width (Fig. 3E, 3F). In the presence of Ag, ILPs were similar in morphology and size (depth = 437 nm, width = 277 nm), but tended to form in denser clusters (Fig. 3E–G). An important feature of the ILPs, whether in the absence or presence of Ag, was the existence of zones of extremely close T cell–EC apposition, typically at the tips of the ILPs as if driven by ILP extension (Fig. 3F, 3G, arrows). Although not exclusive to these locations, quantitative analysis revealed that intercellular contacts of <20 nm were 9-fold greater at ILP tips compared with other regions. The idea that ILPs can exert significant force is supported by their ability to both drive

![FIGURE 4.](http://www.jimmunol.org/) Ag-stabilized ILPs are foci for immune signaling. iTmem cells were incubated with activated, Ag-pulsed (TSST/SEB) HLMVEC for 5 min, fixed, and stained as indicated and imaged by confocal microscopy. (A) Ag-stabilized ILPs (F-actin, green) protrude into MHC-II (red)–enriched podo-prints. Arrows indicate MHC-II–enriched podo-prints. (B) Samples as in (A) were stained for MHC-II (HLA-DR/DQ/DP; red) and MHC-I (HLA A/B/C; green). Schematic of gated regions of interest is shown on the left and included a region outside of the IS (I), the ILP-rich region of the IS (II), and the central region of the IS (III). Right panels show pixel fluorescence intensity histograms for regions I–III. (C) ILPs (F-actin, blue) protruding into podo-prints (mem-YFP, green) are enriched in CD3 (red). Within individual ILPs, CD3 is predominantly focused at the tip (b1) and, to a lesser extent, the edge (b2). (D) PKC-θ (red) is enriched with CD3 (green) in ILPs. (E–G) ILPs (F-actin, blue) colocalize with ZAP70 (E; red), phosphotyrosine (F; red), and HS1 (G; red). (H) HS1 (red) is highly enriched in ILPs. Cross-sectional views from serial-section confocal microscopy are shown of a lymphocyte adherent to the endothelium-presenting Ag. See three-dimensional rotation in Supplemental Video 6. (I and J) ILP arrays were allowed to form as above with the additional presence of physiologic laminar fluid shear flow (2.0 dyne/cm²; arrow indicates direction). (I) MHC-II (red) and F-actin (green) are shown. (J) CD3 (red) and ICAM-1 (green) are shown. Scale bars, 5 μm.
FIGURE 5. T cell ILP formation precedes and supports efficient Ag recognition. (A) Tmem were labeled with Fura-2 and imaged live (at a maximal temporal resolution of 10 s) during migration on mem-DsRed–transfected, Ag-pulsed HLMVECs. Upper panels show mem-DsRed. Arrows indicate initial ILP formation. Middle panels indicate calcium flux values on a rainbow scale. Lower panels provide a schematic representation of newly formed (green) and previously formed (in relation to previous field; red) podo-prints. (a) Frame of initial ILP/podo-print. (b) Frame when calcium flux rises above background. (c) Frame when the peripheral ILP array is stabilized. Note this correlates with peak calcium flux. See also (Figure legend continues).
transcellular diapedesis (34) and to displace/deform organelles such as the nucleus (Fig. 3F).

**Ag-stabilized ILPs share features of traditional TCR signaling microclusters**

To address how these ILP-dominated cell–cell interfaces relate to TCR signaling, we stained for traditional IS markers (15, 20, 21). Confocal microscopy revealed that podo-prints on endothelium were modestly, but consistently, enriched in MHC-II compared with MHC-I or mem-YFP (Fig. 4A, 4B, Supplemental Fig. 3A), whereas T cell ILPs were enriched in CD3 (Fig. 4C, Supplemental Fig. 3B). Comparable enrichment formed under physiologic shear flow (Fig. 4I, 4J). In <5% of T cells, CD3 was distributed into a central supramolecular activation cluster-like cluster rather than in peripheral ILPs (Supplemental Fig. 3C).

Molecules implicated in TCR signaling, including PKC-θ (Fig. 4D, Supplemental Fig. 3D), ZAP70 (Fig. 4E), and phosphorytrosine (Fig. 4F) were also enriched in ILP cores, suggesting active signaling at these sites. The cortactin homolog HS1, a known regulator of both podosomes and ISs (38, 42–44), showed particularly strong enrichment (Fig. 4G, 4H, Supplemental Video 6). Alternatively, the inhibitory molecules CD43 and CD45 localized primarily to the region outside of the T cell–EC interface (Supplemental Fig. 3B). These observations suggest that ILPs may serve as discrete loci for TCR signaling outside of the T cell–EC interface (Supplemental Fig. 3B). To determine whether calcium flux was sufficient to stabilize ILPs, iTmem were incubated on ECs in the absence of Ag, followed by application of thapsigargin to directly increase intracellular calcium. This caused iTmem already engaged in dynamic ILP probing to arrest migration and stabilize ILP clusters (Fig. 6D–G). Thus, whereas dynamic ILP probing of ECs by iTmem seems to facilitate initial Ag recognition, the resulting increase in calcium is both necessary and sufficient to drive accumulation/stabilization of ILPs into podo-synapses.

**ILPs support efficient Ag recognition and sustained signaling**

To determine the functional relationship between ILP formation and T cell activation, we concomitantly monitored calcium flux and ILP dynamics. In the presence of Ag, calcium flux was evident shortly after the first appearance of ILPs. It then peaked several minutes later and gradually decayed over the following 5–60 min (Fig. 5A, 5B, Supplemental Video 7). Calcium flux always occurred subsequent to (or in the same 10-s interval as) the appearance of at least one ILP, with an average offset time of ~25 s (Fig. 5C). In turn, stabilized ILP arrays and symmetric lamellipodia became evident in the ~30–60 s following the increase in calcium (Fig. 5A, 5B, Supplemental Video 7).

These observations suggested that the close intercellular contacts driven by ILPs may promote initial Ag recognition and TCR triggering. Testing this hypothesis is challenging because both ILPs and TCR signaling are fundamentally dependent on F-actin assembly and many of the same actin regulatory pathways (34, 35, 38, 42–44, 48). Thus, we simply compared effects of F-actin inhibition (via latrunculin A) on T cell activation through an Ag-pulsed APC versus direct TCR cross-linking. When stimulated by Ag-pulsed ECs, iTmem pretreatment with latrunculin A caused 100% blockade of both ILP formation and calcium flux during the first 5 min of coincubation (Fig. 5D, 5E). By contrast, when TCRs were stimulated directly by anti-CD3/CD28 cross-linking, significant (although attenuated by ~60%), immediate calcium flux was elicited in the presence of latrunculin A (Fig. 5F, 5G), as shown previously (49). From this we conclude that the total latrunculin A-induced blockade of early response to Ag-pulsed ECs reflects a defect in initial Ag recognition/TCR triggering (Fig. 5F, 5G). We speculate that ILP formation may represent the latrunculin A-sensitive process behind this defect.

**Calcium flux is necessary and sufficient for Ag-mediated ILP stabilization**

Next, we investigated the transition from dynamic ILP probing to formation of stable ILP arrays. Given the correlation between increase in calcium and appearance of ILP arrays, we hypothesized that calcium may be key for stabilizing ILPs. To test this, we pretreated T cells with the calcium chelator BAPTA and the CRAC channel inhibitor BTP2. On Ag-pulsed ECs, this greatly inhibited calcium flux in iTmem, which correlated with strong reduction in the number of stabilized ILPs (Fig. 6A–C). To determine whether calcium flux was sufficient to stabilize ILPs, iTmem were incubated on ECs in the absence of Ag, followed by application of thapsigargin to directly increase intracellular calcium. This caused iTmem already engaged in dynamic ILP probing to arrest migration and stabilize ILP clusters (Fig. 6D–G). Thus, whereas dynamic ILP probing of ECs by iTmem seems to facilitate initial Ag recognition, the resulting increase in calcium is both necessary and sufficient to drive accumulation/stabilization of ILPs into podo-synapses.

The earlier findings point to a positive feedback loop for sustaining TCR signaling. To break this loop, we sought to block proximal signaling downstream of calcium flux, specifically targeting CAMKII via the inhibitor CK59 (50). Treatment with CK59 attenuated Ag-mediated ILP stabilization by ~50% (Fig. 6H), which was coupled to a proportional decrease in calcium flux (Fig. 6I). This result supports the interdependence between ILPs and calcium flux.

**CD8 CTLs use ILPs to probe for Ag**

The endothelium has been shown to initiate MHC-I/Ag-dependent activation of CD8 CTLs leading to direct killing of ECs (31, 32). To investigate these responses, we incubated previously activated murine OT-I CD8 T cells on murine heart microvascular ECs pulsed with SIINFEKL peptide Ag. Ag-pulsed ECs initiated a rapid calcium flux (Fig. 7A) that was coupled to progressive specific lysis of ECs over a 4-h duration (~50% lysis ~2 h; Fig. 7B). Live-cell imaging showed that in the absence of Ag, CTLs avidly probed the endothelium with dynamic ILPs while migrating without fluxing calcium (Fig. 7C, Supplemental Video 8). In the presence of Ag, initial ILPs were rapidly followed by calcium flux (offset time = 32.8 ± 6.6 s), which then led to migrational arrest and formation of peripheral ILP arrays similar to those formed by CD4 effector T cells (Fig. 7D, 7E). Thus, T cell ILPs seem to be a general feature of Ag sensing on ECs.

**Supplemental Videos**

- **Video 7.** Graphical representation of calcium flux with frames a–c noted. (C) Quantitation of ILP-calcium flux offset time. Live-cell imaging was as in (A) and offset time (time from when first ILP forms until calcium flux rises above background) was calculated. Data were binned into 10-s intervals, and average ± SEM is shown for 35 individual T cells from 3 separate experiments. (D) Imaging was performed as in (A) with additional pretreatment of T cells with latrunculin A before addition to Ag-pulsed EC monolayers. (E) Podo-print/ILP index (average number of podo-prints/ILPs per cell) and average calcium flux at 5 min was calculated. Both analyses are pooled mean ± SEM from three separate experiments. (F) iTmem were labeled with Fluo-4 and imaged live with anti-CD3/CD28 cross-linking with or without latrunculin A pretreatment. Left panels show resting T cells. Right panels show activated T cells imaged 60 s after addition of cross-linking Abs. Arrows indicate de novo formation of micron-scale T cell protrusions. (G) Average calcium flux was calculated from three separate experiments as in (F). Scale bars, 5 μm. Data represent mean ± SEM. *p < 0.05, **p < 0.0005.
A planar-coated cell model for Ag recognition

We next considered whether ILPs either represent unique features of T cell-endothelial Ag recognition or may be more broadly relevant and uniquely revealed by endothelium. We speculated that the rigidity of coated substrate models may frustrate/mask ILP formation, whereas orientation/resolution issues might obscure detection of ILPs with traditional cellular APCs (16–18).

First, we simply asked whether evidence consistent with ILPs could be detected in a coated glass model (Fig. 8A). IT_{mem} plated on glass coated with ICAM-1 and anti-CD3 (but not anti-CD43/CD45) Ab rapidly fluxed calcium (Supplemental Fig. 4A–C). IRM revealed that initial “microcontacts” (~0.2- to 1-μm dots consistent with T cell ILPs and/or microvilli) always preceded calcium flux (Supplemental Fig. 4B, 4C; offset time = 21.4 s). New microcontacts also continued to form after T cell spreading (Fig. 8B, Supplemental Fig. 4D, 4E, Supplemental Video 9).

We hypothesized that these microcontacts at least partly reflected ILP activity that was mechanically frustrated by the rigid substrate. To test this, we designed a “coated-cell APC” model whereby the earlier Ab-coated glass substrate was recapitulated on the surface of a deformable CHO-K1 cell (Fig. 8C, Supplemental Fig. 4F). In this setting, clear three-dimensional ILP arrays were readily detected that were coupled to calcium flux (Fig. 8D, Supplemental Video 10).

FIGURE 6. Calcium flux is necessary and sufficient for ILP stabilization. (A) IT_{mem} were Fura-2 labeled, pretreated with the calcium chelator BAPTA and the CRAC channel inhibitor BTP2, and imaged live on Ag-pulsed ECs. Arrows indicate a few sporadically formed podo-prints. (B and C) Podo-print/ILP index (B) and average calcium flux (C) were calculated at 5 min for experiments as in (A). (D) Live-cell imaging was conducted in the absence of Ag before and after addition of the calcium ionophore thapsigargin (at time = ‘0:00’). Arrows indicate ILPs/podo-prints. (E) Correlation of calcium flux with ILP number after addition of thapsigargin. (F) Quantitation of the lifetime of ILPs with or without addition of thapsigargin. (H and I) Imaging and analysis was performed as in (A)–(C) except T cells were pretreated with the CAMKII inhibitor CK59. Data represent mean ± SEM. Scale bars, 5 μm. **p < 0.005, ***p < 0.0005.
ways preceded (Supplemental Fig. 4G; offset time ∼56 s) and seemed to functionally support (Fig. 8E, 8F) initiation of calcium flux. These findings suggest a general tendency of memory/effector T cells to use ILPs to probe diverse substrates for recognition and response to Ag (as modeled in Fig. 9).

ILPs are involved in recognition of Ag presented by professional APCs

Next, we re-examined Ag recognition with professional APCs. Thus, iTmem were incubated with Priess B cells pulsed with Ag and imaged by confocal microscopy. Equatorial cross sections recapitulate classic views (12) of the T cell–B cell IS whereby T cell LFA-1 shows enrichment in peripheral-supramolecular activation cluster–like peripheral membrane bulges (Supplemental Fig. 4H, inset 2, arrows). Cross sections taken at the edge of the IS additionally suggest presence of LFA-1–rich finger-like protrusions (Supplemental Fig. 4H, insets 1a–d) similar to ILPs formed on ECs (Fig. 3Bb). More compelling imaging of putative ILPs can be seen in occasional examples where the T cells settle on top of B cells such that the IS aligns with the optimal imaging plane (18) (Fig. 8G, Supplemental Video 11). Ultrastructural views, evident in a subset of micrographs, further support the presence of ILPs (Fig. 8H).

Finally, we investigated murine OTII CD4 T cells incubated with BMDCs coexpressing mem-YFP and soluble DsRed. T cells interacted with DCs both laterally (Fig. 8I, subset a) and to a lesser extent through en face contacts (Fig. 8I, subset b). In the former, side views of DC invaginations were readily evident that were similar to podo-prints/ILPs seen on endothelium (Fig. 8I, panel 3i). En face interactions also revealed discrete circular podo-prints/ILPs (Fig. 8I, panels 3ii, 3iii), although these were generally less obvious than those formed on endothelium because of the highly active and irregular surface topology of DCs. Dynamic imaging showed ILP probing preceded calcium flux, which, in turn, was coupled to peripheral ILP array formation (Fig. 8J). Ultrastructural confirmation of ILP formation could also be obtained.
FIGURE 8. ILPs facilitate Ag recognition on professional APCs. (A) Schematic of the “coated-glass model” using ICAM-1– and Ab-coated glass. (B) Magnified view of the interaction surface. (B) iTmem were added to an ICAM-1–Fc– and anti-CD3–coated glass chamber and imaged by differential interference contrast and IRM. Arrows indicate IRM-detected “microcontacts.” (C) Schematic of the “planar coated-cell APC model.” CHO-K1 cells expressing ICAM-1–GFP and soluble DsRed were surface “coated” with Abs against CD3, CD43, or CD45 using a biotin/SA capture approach (see also Supplemental Fig. 4F). (D) Magnified view of the interaction surface. (D) Cells prepared as in (C) coated with anti-CD3 Ab were (Figure legend continues)
FIGURE 9. Hypothetical model for ILP function in Ag recognition and response. Schematic depicts top-down and side views of a memory/effector T cell interacting with an APC/target cell. Lymphocytes initiate lateral migration (Step 1) and begin to dynamically drive ILPs against the apposing cell (Step 2, a). Close interactions between T and APC/target cells, which are partially opposed by the cell glyocalyces (2b), form preferentially (but not exclusively) at ILP tips (2c). We hypothesize that TCR/MHC interactions may be facilitated in these zones. Initial calcium released on Ag recognition (green overlay/arrows; Step 3) seems to be coupled to stabilization/accumulation of ILP arrays ("podo-synapses"; Step 4), which we hypothesize could, in turn, help sustain/enhance signaling (Step 5).

in a subset of electron micrographs (Fig. 8K). Finally, treatment of T cells with latrunculin A strongly inhibited both ILPs and initial calcium responses to Ag presented by DCs (Fig. 8L).

Discussion
Basic cellular mechanisms by which lymphocytes effectively scan for peptide Ag on apposing cells and how this leads to IS formation have remained mysterious. Our results investigating endothelium as a model APC indicate potential roles for ILPs in these processes. Our further studies with diverse Ag recognition settings suggest broad, previously unappreciated functions for ILPs as actuators of immune surveillance.

Vascular ECs represent intriguing and understudied APCs/target cells for adaptive immune responses. These MHC-I-, MHC-II-, and costimulator-expressing cells are strategically positioned at the blood–tissue interface to serve as unique sentinels for the immune system (26). Previous studies demonstrated that ECs can effectively restimulate memory/effector, but not naïve, T cells (26–33). Thus, ECs have been hypothesized to serve as peripheral ancillary or "semiprofessional" APCs that contribute to the effector phase of adaptive immune responses. Although overall roles remain controversial, studies suggest that Ag presentation by ECs can influence T cell activation, differentiation, trafficking, and memory (26–28, 51). In this study, we demonstrate that CD4 and CD8 memory/effector T cells exhibited a transient arrest in migration coupled, respectively, to Th1 and cytotoxic responses on ECs presenting MHC/Ag, consistent with previous findings (31, 52, 53).

Importantly, ECs exhibit a virtually planar cell surface in vitro, which we previously established to be ideal for spatiotemporal resolution imaging of topological dynamics (34). We reasoned that ECs might, therefore, serve as physiologic "planar APC/target cell" models uniquely suited to fill two key gaps in our understanding of immune surveillance mechanisms: 1) how initial MHC/Ag-sampling contacts form between T cells and APCs, and 2) how these lead to formation of productive ISs. Although planar APC substrates (i.e., lipid bilayers, Ab-coated glass) provide excellent imaging, which has led to critical insights to these dynamics (15, 19–22), such models are inherently limiting. Alternatively, details of physiologic cell–cell scanning dynamics are profoundly obscured by orientation-related imaging issues (16–18). The imaging afforded with our planar endothelial APC model in this study revealed unexpected involvement of ILPs in Ag recognition and response activities.

ILPs ("invadosome-like protrusions") are lymphocyte equivalents (36) to podosomes (formed largely by myeloid lineage cells) and invadopodia (formed by transformed cells), collectively termed "invadosomes" (35). Invadosomes and ILPs are actin-dependent cylindrical, protrusive organelles (∼200–1000 nm in diameter and depth) that form on the ventral surface of migratory cells (35). Distinct from invadosomes, characterized to form on matrix, bone, glass/plastic substrates, T cell ILPs have only been seen on cellular substrates, as shown in a range of in vitro (34, 54, 55) and in vivo studies (reviewed in Ref. 56). In such settings, dynamic ILP probing seems to function in migratory pathfinding.
apparently serving as biomechanical sensors (34, 36, 54, 55, 57). These studies suggest that ILPs may also facilitate biochemical or “informational” scanning of diverse cell surfaces.

We speculated that the ILP scanning seen on ECs was uniquely revealed by this setting, rather than a unique feature of it. IRM imaging of a rudimentary APC model (anti-CD3–coated glass) showed that T cells form a series of microcontacts with the substrate that are consistent with either ILP or microvilli interactions. Similar microcontacts were previously shown to be enriched in F-actin, WASP, and TCR, and hypothesized to represent frustrated podosomes (20, 22). When we tested this hypothesis by recapitulating the same anti-CD3 activation stimuli on a pliable cell surface, we, indeed, found that the microcontacts transitioned into three-dimensional ILPs and podo-prints. Moreover, our studies using approaches optimized for imaging professional APCs (i.e., B cells and DCs) readily detected ILPs probing in these settings. Importantly, although dynamics, molecular composition, and three-dimensional architectures were not defined, extensive (largely EM-based) studies previously evidenced similar T cell protrusions extending against diverse APCs and target cell surfaces (21, 43, 58–63). Collectively, this suggests broad relevance for memory/effector T cells probing their cellular environment with ILPs. The important question of whether naive T cells similarly use ILPs remains to be addressed.

One possible role for ILP probing may be in enhancing the efficiency of Ag recognition. The act of Ag detection requires T cell and APC/target membranes to come within ∼14 nm of each other (2, 64). Yet, all cell membranes are extensively shielded by ∼50- to 500-nm-thick glyocalyx (3–5) that physically oppose close contact and negatively modulate immune recognition (5–10) (Fig. 9, inset 2b). In the periphery, patrolling effector/memory lymphocytes must effectively scan the surfaces of widely varying cell types (indeed, essentially any host cell could become a target), each with glyocalyxes of different thicknesses and physicochemical properties. Forces provided by ILP (which form independently of Ag during lateral migration) (34) are sufficient to deform nuclear lamina, displace and distort cytoskeleton and intracellular organelles, and promote transcellular diapedesis (34). More than sufficient energy would, therefore, seem to be available at the tips of ILPs (Fig. 9, inset 2a) to overcome repulsion provided by the glyocalyx (Fig. 9, inset 2c).

Previous studies showed that lamellipodia of migrating lymphocytes are zones of heightened Ag recognition efficiency (65–67). In both our previous (34) and current work, we show that lamellipodia are also the predominant areas of ILP formation. Moreover, in our experimental system, ILPs, but not necessarily lamellipodia, consistently preceded calcium flux. Thus, although migration behaviors are necessary for immune surveillance, ILPs may function more directly in promoting intimate contacts, and thereby ensuring efficient Ag sampling. Our studies with latrunculin A are consistent with (although do not prove) such functional roles. It is also interesting to note that forces and membrane bending associated with ILPs are consistent with the hypothesized force-based mechanism for formal triggering of TCR signaling (22, 68–71). ILPs are probably not absolutely required for either TCR signaling or forming intimate T cell–APC/target contacts, but rather act to ensure efficiency and fidelity of immune surveillance.

Another possible function for ILPs may be to support signal amplification and sustenance. Our studies reveal dense arrays of calcium-stabilized ILPs that dominate the IS after Ag recognition. Individual ILPs showed enrichment in TCR and molecules suggestive of active signaling. This feature is generally not unlike TCR signaling microclusters defined using planar APC model substrates (14, 15, 20–22, 38, 72, 73), with the key distinction that ILPs have a discrete three-dimensional architecture. The well-developed concept that microclusters may function as “signalosomes” to amplify and sustain signaling by concentrating important molecules/activities might then be extended to include limited “reaction volumes” formed within ILPs (17, 74–78). In addition, the stable peripheral ILP arrays are strikingly similar to osteoclast “podosome-belts” (79, 80) that form sealing zones for directed secretion of bone-degrading enzymes (81). Thus, it may be hypothesized that peripheral podo-synapses may function analogously for directed secretion of cytokines or cytotoxic materials.

These studies collectively support the hypothesis that ILPs may serve as newly appreciated sensory organelles that facilitate Ag recognition and responses. By virtue of physical force exertion, ILPs literally allow T cells to get a deeper understanding of their local cellular environment. Such proactive “informational scanning” might ensure robust sampling of MHC/Ag on diverse cell types. It is tempting to speculate that stabilized TCR-enriched ILPs resulting from Ag recognition represent a clearer, more physiologic view of TCR microclusters and “multifocal” ISs characterized to date.

Disclosures

The authors have no financial conflicts of interest.

References


